

The Basic Domain of Myogenic Basic Helix-Loop-Helix (bHLH) Proteins Is the Novel Target for Direct Inhibition by Another bHLH Protein, Twist

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In vertebrates, the basic helix-loop-helix (bHLH) protein Twist may be involved in the negative regulation of cellular determination and in the differentiation of several lineages, including myogenesis, osteogenesis, and neurogenesis. Although it has been shown that mouse twist (M-Twist) (i) sequesters E proteins, thus preventing formation of myogenic E protein-MyoD complexes and (ii) inhibits the MEF2 transcription factor, a cofactor of myogenic bHLH proteins, overexpression of E proteins and MEF2 failed to rescue the inhibitory effects of M-Twist on MyoD. We report here that M-Twist physically interacts with the myogenic bHLH proteins in vitro and in vivo and that this interaction is required for the inhibition of MyoD by M-Twist. In contrast to the conventional HLH-HLH domain interaction formed in the MyoD/E12 heterodimer, this novel type of interaction uses the basic domains of the two proteins. While the MyoD HLH domain without the basic domain failed to interact with M-Twist, a MyoD peptide containing only the basic and helix 1 regions was sufficient to interact with M-Twist, suggesting that the basic domain contacts M-Twist. The replacement of three arginine residues by alanines in the M-Twist basic domain was sufficient to abolish both the binding and inhibition of MyoD by M-Twist, while the domain retained other M-Twist functions such as heterodimerization with an E protein and inhibition of MEF2 transactivation. These findings demonstrate that M-Twist interacts with MyoD through the basic domains, thereby inhibiting MyoD.

Basic helix-loop-helix (bHLH) transcription factors play central roles in specifying and maintaining cell identity (32, 33, 45, 66). The bHLH motif is defined by the basic domain that mediates specific DNA binding, the HLH domain containing two amphipathic helices that act as dimerization domains, and a nonconserved loop region that separates the two helices (33, 42). The bHLH factors can be classified into several groups based on their tissue distribution, partner choice, DNA-binding properties, and structural features. (i) E proteins, the products of E2 genes (E2A, E2-2, and HEB/HTF4), also called class A, are widely expressed and serve as a heterodimer partner for the tissue-specific class of bHLH proteins (30, 36, 43, 53, 74). (ii) These tissue-specific, class B, bHLH proteins bind E box DNA sequences (CANNTG) (10, 31). Examples of class B factors include the MyoD family of myogenic bHLH proteins, MASH-1, MASH-2, Twist, SCL, and neuroD (1, 37, 44, 63). (iii) Class C members, such as the myc family, have a leucine zipper motif carboxyl (C)-terminal to the bHLH region. (iv) Additional HLH proteins, such as members of the Id family, can form a complex with class A and some class B factors (6). Since Id members lack a basic region, they form a complex incapable of binding to DNA and act as a dominant negative inhibitor for class B factors.

The myogenic bHLH proteins Myf-5, MyoD, myogenin, and MRF4 are required for muscle cell determination and differentiation and activate muscle-specific transcription through several steps: heterodimerization with E proteins, heterodimer binding to specific E box DNA sequences (36, 43), recognition of the basic domain by an as-yet-undefined mechanism (18, 19,

40, 68), and presumably subsequent unmasking of the N-terminal transcriptional activation domain. Myocyte enhancer factor 2 (MEF2) binding sites are frequently present in the promoters and enhancers of many skeletal and cardiac muscle-specific genes, and MEF2 proteins have been shown to cooperatively activate the transcription through direct interaction with myogenic bHLH proteins (46).

In *Drosophila melanogaster*, the class B bHLH protein Twist is involved in the establishment of early mesoderm and later in a choice between alternative mesodermal cell fates (5). Mouse embryos express Twist (M-Twist) in cranial neural crest cells as well as in mesoderm (25, 60, 71). M-Twist expression in somites diminishes as cell-specific differentiation emerges. As newly formed somites differentiate to form compartments, M-Twist expression is excluded first in the myotomal compartment where expression of myogenic bHLH proteins is upregulated (16, 48, 51, 57, 58). Homozygous M-Twist knockout mice fail to close the cranial neural tube and die at embryonic day 11.5 (15). In the somites of these mice, apoptotic cell death is evident, and the individual somitic compartments are poorly partitioned, consistent with the concept of a role for M-Twist in the prevention of premature differentiation of cells and in the compartmentalization of a somite. Cultured C2 muscle cells, when stably transfected with M-Twist expression vectors, lose the ability to differentiate, with concomitant loss of expression of the myogenic bHLH proteins (28). Taken together, these findings suggest that vertebrate Twist may be involved in the negative regulation of cellular determination and in the differentiation in the muscle cell lineage.

It has been shown that M-Twist inhibits MyoD transactivation of the muscle creatine kinase (MCK) enhancer/promoter through two mechanisms (59): (i) E protein sequestration, which would prevent formation of E protein-MyoD complexes

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and (ii) the inhibition of MEF2 transactivation through the direct interaction mediated by the C terminus of M-Twist. However, overexpression of both E protein and MEF2 failed to rescue the M-Twist inhibition of MyoD transactivation (27a), suggesting additional mechanisms for the inhibition by M-Twist. In the present study, we provide evidence that M-Twist directly interacts with the myogenic bHLH proteins. Unlike conventional heterodimerization mediated by HLH domains, the novel interaction between M-Twist and MyoD requires the basic domains of both proteins as essential components. This is the first example of protein-protein interaction among the members of class B-type bHLH proteins. Several M-Twist mutants incapable of binding to MyoD failed to inhibit MyoD transactivation but maintained other functions, including heterodimerization with E12 and inhibition of MEF2 transactivation from an MEF2-dependent promoter. These findings demonstrate that M-Twist interacts with MyoD through a novel mechanism mediated by the basic domains and that this interaction is essential for M-Twist inhibition of MyoD transactivation.

MATERIALS AND METHODS

Cells, transfections, and CAT assay. C3H10T1/2 mouse embryo fibroblasts were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated fetal calf serum. For transient transfections, approximately 50% of the confluent cells in 6-cm-diameter dishes were refed 1 to 3 h prior to addition of the DNA as a calcium phosphate precipitate, which is prepared with *N,N*-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid (BES)-buffered saline containing a solution of 50 mM BES (pH 6.95), 280 mM NaCl, and 1.5 mM Na₂HPO₄ (14). A total of 9 μ g of plasmid DNA per dish was used for transfections. The precipitate was aspirated 20 to 24 h later, and the cells were refed with differentiation medium consisting of DMEM with 2% horse serum. The cells were incubated for an additional 2 days and harvested for CAT assay as described previously (55). All CAT assays were done twice in at least three independent experiments and normalized to total protein in extracts prior to acetylation reactions. An AMBIS dual radioanalytic imaging system was used to determine the amount of ¹⁴C reaction products and substrate from thin-layer chromatographic plates.

Plasmids. M-Twist cDNA was generously provided by B. Wold (California Institute of Technology). MCK-CAT has been described previously (54). The expression constructs for MyoD, MyoD- Δ N, MyoD-E12B, MyoD-mycB, MyoD-T4B, and GST-MyoD were kindly provided by S. Tapscott, R. Davis, and H. Weintraub (Fred Hutchinson Cancer Center). The cDNAs for myogenin and MEF2C were provided by E. N. Olson (University of Texas Southwestern), Myf5 was provided by H.-H. Arnold (Institut für Biochemie und Biotechnologie, Braunschweig, Germany), and MRF4 was provided by S. Konieczny (Purdue University). M-Twist, MyoD, and MEF2C were subcloned into pcDNA3 (Invitrogen) (CMV-M-Twist, -MyoD, and -MEF2C). The M-Twist Δ Bpu mutant was created by digesting the CMV-M-Twist with Bpu1102I, which excises the region containing the loop and helix 2, and religating the vector. All the other M-Twist mutants were created by PCR and contained a *Bam*HI site, the Kozak sequence prior to the initiation codon, and a stop codon and an *Eco*RI site at the C terminus. The DNA fragments were cloned into the *Bam*HI-*Eco*RI site of pcDNA3, and the sequences were verified. For CMV-Twist-bHLH and -HLH, a nuclear localization signal (RRKRR) originally present in the N terminus of wild-type M-Twist was introduced to the N termini. Myc epitope-tagged M-Twist clones were created by subcloning the various M-Twist mutants into pcDNA3-MT, a derivative of pCS2+MT (59). GST-E12 has been described previously (54). For GST-Twist, an *Afl*III-*Sst*I fragment was isolated from Bluescript II-Twist, end filled, and inserted into the *Sma*I site of pGEX-2TK (Pharmacia). GST-MyoD-bHLH, GST-MyoD-HLH, GST-MyoD-bH1, GST-Twist-bHLH, and GST-Twist-HLH were created by PCR with *Pfu* DNA polymerase (Stratagene). The resulting DNA segments were inserted into *Bam*HI-*Eco*RI sites of pGEX-2TK in frame, and the sequences were verified. For immunoprecipitation experiments, the HA tag sequence was introduced at the amino terminus of M-Twist by PCR (CMV-HA-M-Twist), and the FLAG tag sequence was introduced into the C terminus of MyoD (CMV-MyoD-FLAG).

In vitro transcription and translation. In vitro transcription and translation were carried out with TNT coupled reticulocyte lysate systems (Promega) and L-[³⁵S]methionine (>1,000 Ci/mmol; Amersham) according to the manufacturer's protocol.

In vitro protein interaction studies. The GST fusion proteins were prepared as described elsewhere (56). For the protein-protein interaction assays, comparable amounts of resin-bound GST fusion proteins were incubated with 10 μ l of in vitro-translated proteins in NETN buffer (0.5% Nonidet P-40-1 mM EDTA-20

mM Tris-HCl [pH 8.0]-100 mM NaCl-2 μ g of aprotinin per ml-2 μ g of leupeptin per ml-1 μ g of pepstatin per ml-10 μ M 4-aminodiphenylmethane-sulfonyl fluoride [APMSF]) containing 25 μ g of ethidium bromide per ml. After 1 h of incubation at 4°C, the resins were washed thrice with 1 ml of ice-cold NETN and the bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then fixed, incubated in Enlightening solution (Du Pont) for 30 min, dried, and exposed for fluorography.

Immunoprecipitation. COS7 cells (American Type Culture Collection) were cultured to approximately 70% confluency in 10-cm-diameter dishes. The cells were transfected in BES-buffered saline as described above with either control vectors or CMV-HA-M-Twist and CMV-MyoD-FLAG. After 16 h at 3% CO₂, the cells were refed with growth medium and incubated at 10% CO₂ for 36 h. For labeling, the cells were rinsed twice with phosphate-buffered saline (PBS) and incubated for 30 min in methionine-free DMEM. The cells were then labeled with 0.37 mCi (>1,000 Ci/mmol) of L-[³⁵S]methionine (Amersham) in methionine-free DMEM with 15% dialyzed fetal calf serum. After 3 h, the cells were rinsed four times with ice-cold PBS, incubated in PBS with 10 mM EDTA at 4°C for 10 min, and harvested. The cells were centrifuged at 4°C, and the pellets were lysed by incubation at 4°C for 1 h in nuclear lysis buffer (20 mM HEPES [pH 7.7]-20% glycerol-10 mM NaCl-1.5 mM MgCl₂-0.2 mM EDTA-0.1% Triton X-100-1 mM dithiothreitol-10 μ M APMSF-1 μ g of pepstatin per ml-100 μ g of aprotinin per ml). The lysate was centrifuged in an Eppendorf centrifuge at 4°C for 5 min, and the supernatant was recovered. An aliquot with radioactivity of approximately 6 \times 10⁷ cpm was incubated in 400 μ l of RIPA buffer (10 mM Tris-Cl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, 0.5% Nonidet P-40, and 10 μ M APMSF) and incubated with a first antibody coupled to protein G-agarose (Sigma), which was prepared as follows. One microliter (3 μ g) of anti-FLAG M2 antibody (Kodak) was preadsorbed to 30 μ l of a 50% slurry of protein G-agarose for 3 h at 4°C and washed thrice. The radiolabeled nuclear extract was incubated with the resin-bound antibody for 3 h at 4°C in the presence of 25 μ g of ethidium bromide per ml in RIPA buffer. The resins were washed thrice with the same buffer, and the bound proteins were released by incubating the resins at 90°C for 10 min. The supernatant was recovered and incubated with the HA antibody, which had been coupled similarly to the protein G-agarose. After 2 h at 4°C in RIPA buffer, the resins were washed thrice and the bound proteins were fractionated by SDS-PAGE.

RESULTS

M-Twist interacts with MyoD in vivo. We determined whether M-Twist interacted with MyoD within cells, using HA-tagged M-Twist and FLAG-tagged MyoD. We first confirmed that the two antibodies used for sequential immunoprecipitation do not cross-react with the each other's antigens. When labeled lysate from COS cells transfected with only the MyoD-FLAG vector was immunoprecipitated with FLAG antibody, a major band representing MyoD was detected (Fig. 1A, lane 1, band a). However, subsequent incubation with the HA antibody did not precipitate any detectable proteins (Fig. 1A, lane 2). Similarly, when the lysate from cells transfected with only the HA-Twist vector was incubated with the HA antibody, HA-Twist was specifically precipitated (Fig. 1B, lane 2, band b). However, when the same lysate was first incubated with FLAG antibody (Fig. 1C, lane 1) and subsequently with the HA antibody, no bands were detected (lane 2), confirming no cross-reactivity under these experimental conditions.

Therefore, the cells were next cotransfected with both MyoD-FLAG and HA-Twist vectors, and the labeled lysates were incubated with FLAG antibody (Fig. 1D, lane 4). Three major bands, a (MyoD-FLAG), b (HA-Twist), and c, were detected. The identity of band c is unknown. The interaction of anti-FLAG antibody with MyoD-FLAG is highly specific, since band a (MyoD-FLAG) was not predominant amidst the entire population of labeled proteins, and no differences in banding patterns were observed between the control (Fig. 1D, lane 2) and the MyoD-FLAG-transfected cell lysates (Fig. 1D, lane 3). When challenged with FLAG peptide, the intensity of all three bands, a, b, and c, but not of the other nonspecific bands, such as d, was markedly attenuated (Fig. 1D, lane 5), suggesting that bands a, b, and c were specifically precipitated by the FLAG antibody. Both bands a (MyoD-FLAG) and c are doublets, and the slower-migrating protein of each doublet proved to be a phosphorylated form of the faster-migrating ones, since treat-

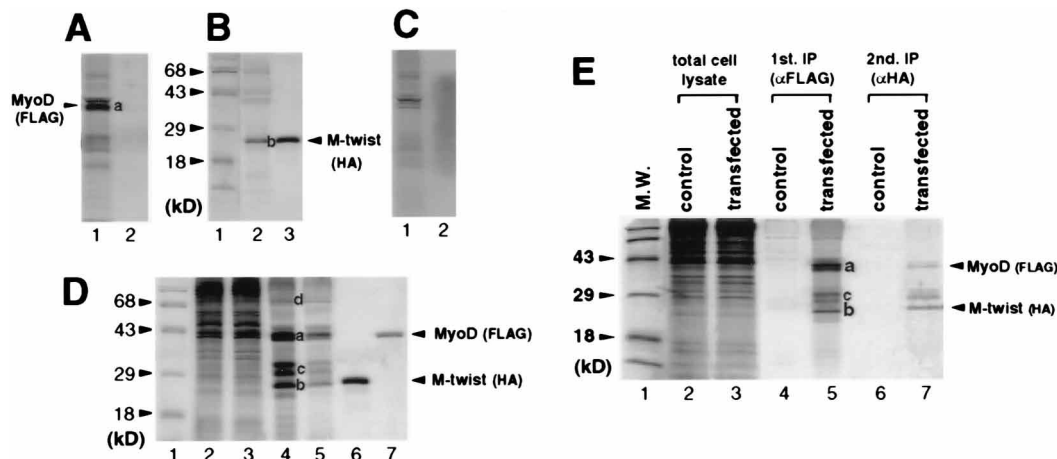


FIG. 1. MyoD and M-Twist interact with each other in cells. (A) COS cells were transiently transfected with only CMV-MyoD-FLAG (10 μ g), and the cell lysate was immunoprecipitated with anti-FLAG M2 antibody (lane 1). The precipitated proteins were released and subjected to reprecipitation with the HA antibody (lane 2). No bands were detected. (B) The cells were transfected with only CMV-HA-Twist, and the lysate was precipitated with the HA antibody (lane 2). Lane 1, 14 C-labeled protein molecular size markers; lane 3, in vitro-translated HA-Twist. (C) The cells were transfected with only CMV-HA-Twist. The lysate was first incubated with the FLAG antibody (lane 1). Several faint nonspecific bands were observed. The precipitated proteins were released and reincubated with the HA antibody (lane 2). No bands were detected. (D) COS7 cells were transfected either with 20 μ g of control vector alone (lane 2) or with both 10 μ g of CMV-MyoD-FLAG and 10 μ g of CMV-HA-Twist (lanes 3 to 5), and cell lysates were subjected to immunoprecipitation with the FLAG antibody. Lane 1, marker peptides; lanes 2 (control) and 3 (MyoD-FLAG plus HA-M-Twist), total cell lysates; lanes 4 and 5, proteins precipitated from the lysate by the FLAG antibody in the absence (lane 4) or presence (lane 5) of 1 μ g of FLAG peptide. Bands a (MyoD-FLAG), b (HA-M-Twist), and c precipitated by an anti-FLAG antibody (lane 4) are equally diminished by the FLAG peptide, while other nonspecific bands, such as d, are not (lane 5). Lanes 6 and 7, in vitro-translated HA-Twist and MyoD-FLAG. (E) COS7 cells were either transfected with 20 μ g of control vector alone (lanes 2, 4, and 6) or cotransfected with 10 μ g of CMV-MyoD-FLAG and 10 μ g of CMV-HA-Twist (lanes 3, 5, and 7). Lane 1, 14 C-labeled protein molecular size markers (in kilodaltons); lanes 2 (control) and 3 (MyoD-FLAG plus HA-Twist), total cell lysates; lanes 4 and 5, proteins precipitated from either the control lysate (lane 4) or the one containing both MyoD-FLAG and HA-Twist (lane 5) by the FLAG antibody (α FLAG). In lane 5, the MyoD-FLAG (a), HA-Twist (b), and a doublet of unknown identity (c) are indicated. The bound peptides were released by incubation at 90°C for 10 min and reprecipitated with the HA antibody (α HA) (lane 6, control; lane 7, CMV-MyoD-FLAG- and CMV-HA-Twist-transfected cells). IP, immunoprecipitation.

ment with calf intestine phosphatase diminished the slower bands (not shown).

Under these experimental conditions, we next incubated the labeled lysates from the cells cotransfected with both MyoD-FLAG and HA-Twist vectors, first with the FLAG antibody and, for a second precipitation, with the HA antibody. The bands a, b, and c, present after anti-FLAG immunoprecipitation (Fig. 1E, lane 5), were each reprecipitated by HA antibody (Fig. 1E, lane 7), with the highest efficiency for band b (HA-Twist). No labeled proteins were immunoprecipitated from the control lysate after the second precipitation (Fig. 1E, lane 6). These findings indicate that M-Twist physically interacts with MyoD within cells and that an unknown peptide (c) may also be present in the MyoD-M-Twist complex.

M-Twist interacts directly with all four myogenic bHLH proteins in vitro. If M-Twist directly interacts with MyoD, then this interaction should be able to be reconstituted in vitro in the absence of cellular bridging proteins that might mediate the M-Twist-MyoD interaction. To address this issue, we performed in vitro protein-protein interaction studies, using bacterially expressed GST-M-Twist fusion protein and in vitro-translated MyoD as well as E12. First we confirmed that M-Twist interacts with E12 and MyoD in vitro (Fig. 2, lanes 1 and 3). It has been reported in *Drosophila* that Twist may homodimerize (63). Accordingly, we tested this possibility for M-Twist and found that M-Twist can also homodimerize in vitro (Fig. 2, lane 5). If the M-Twist-MyoD interaction is mediated by a MyoD domain conserved in the myogenic bHLH proteins, then this interaction should be observed with the other members of the family as well. Indeed, the interaction between M-Twist and the myogenic bHLH proteins is not limited to MyoD but is observed also for the other three members, including myf5, myogenin, and MRF4 (Fig. 2, lanes

10 to 12). M-Twist, however, did not interact with the bHLH leucine zipper class C Max protein (not shown), consistent with previous observations that class B and C proteins do not interact. The interaction between M-Twist and MyoD was also confirmed in a reciprocal experiment with GST-MyoD and 35 S-labeled M-Twist (see Fig. 3A, lane 7). These findings suggest that M-Twist interacts with the myogenic bHLH proteins, presumably by recognizing a specific conserved domain or structure present in this family of proteins.

The basic domain of MyoD mediates the interaction with M-Twist. Since M-Twist inhibits MyoD transcriptional activity, we hypothesized that the MyoD transcriptional activation domain residing in the amino (N) terminus might be a direct

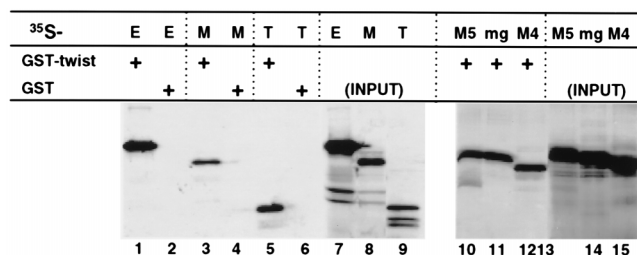
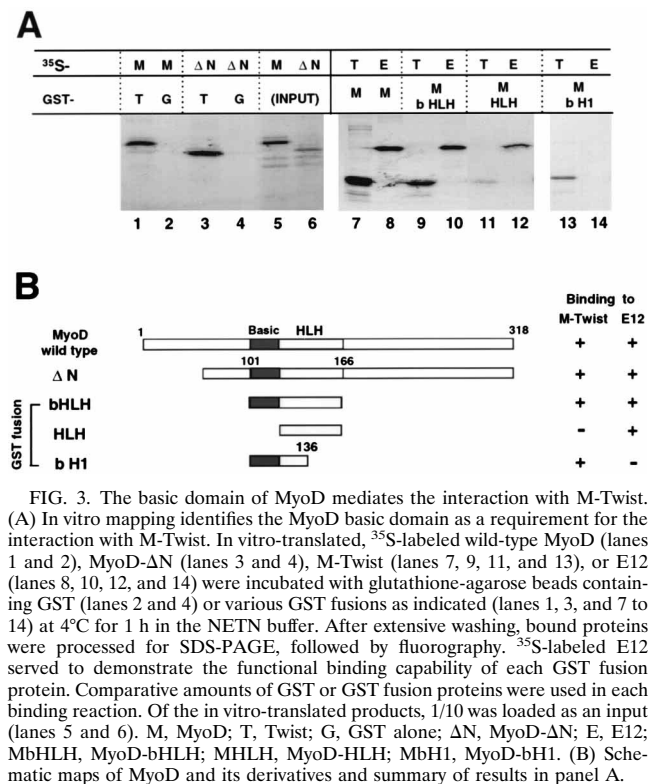


FIG. 2. M-Twist physically interacts with the myogenic bHLH transcription factors in vitro. In vitro-transcribed-and-translated, 35 S-labeled E12 (lane 1 and 2), myogenic bHLH proteins (lanes 3, 4, and 10 to 12), or M-Twist (lanes 5 and 6) were incubated with glutathione-agarose beads containing GST-Twist (lanes 1, 3, 5, and 10 to 12) or GST alone (lanes 2, 4, and 6) and incubated at 4°C for 1 h in NETN buffer. After extensive washing, bound proteins were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was directly loaded onto the gel as an input (lanes 7 to 9, 13 to 15). E, E12; M, MyoD; T, Twist; M5, myf-5; mg, myogenin; M4, MRF4.



target for M-Twist inhibition. Deletion of the N-terminal activation domain of MyoD, however, did not affect the interaction with M-Twist (Fig. 3A, lane 3), suggesting that the MyoD activation domain does not play a primary role in the physical interaction with M-Twist. We next focused on the bHLH domain, a known protein interaction domain for bHLH proteins in general. We found that the isolated MyoD bHLH domain (MyoD-bHLH) retained strong binding activity to M-Twist (Fig. 3A, lane 9). Since the bHLH domain can be separated into two functionally distinct regions, a basic domain for DNA binding and an HLH domain for protein dimerization, we sought to determine whether the HLH domain alone without the basic domain could mediate the interaction. Surprisingly, we found that the isolated HLH domain of MyoD cannot support the interaction with M-Twist (Fig. 3A, lane 11), even though the HLH domain is properly folded, as indicated by its interaction with E12 (lane 12). Although the data clearly suggest that M-Twist-MyoD interaction cannot be mediated by a conventional HLH-HLH domain interaction strategy (33, 42), it was unclear whether the MyoD basic domain directly contacts M-Twist. However, we found that the truncated MyoD polypeptide, consisting of only 35 amino acids of basic region and helix 1 in GST fusion, can confer the interaction with M-Twist (Fig. 3A, lane 13), while the interaction with E12 was completely abolished (lane 14). This finding and the inability of helix 1 of the HLH domain (lane 11) to interact with M-Twist strongly suggest that the MyoD basic domain directly contacts M-Twist and thus represents a novel mode of protein-protein interaction among the cell type-specific class B bHLH proteins.

The MyoD HLH domain contributes to the interaction with M-Twist. Although the above-described findings indicate that the basic domain is involved in the interaction, other domains may also contribute to it. We focused on the HLH domain of MyoD and studied the effects of several mutations in the HLH

region. The MyoD swap mutants, mycH1 and mycH2, have helix 1 or 2 replaced by those from mouse c-myc protein (Fig. 4B). These MyoD mutants are unable to heterodimerize with E proteins (Fig. 4A, lanes 6 and 10), presumably because they cannot form proper HLH structures (18). Interestingly, we found that these mutants also lost the ability to interact with M-Twist (Fig. 4A, lanes 5 and 9), even though the isolated bH1 fragment can (Fig. 3A, lane 13). This indicates that the interaction between MyoD and M-Twist depends on a proper HLH structure in the whole MyoD molecule background. The basic domain might be buried within the whole molecule in the absence of proper protein folding conferred by the aberrant HLH domain, whereas the isolated bH1 fragment is more likely to be free of such constraints. The requirement for a properly structured HLH domain was further confirmed with more subtle point mutations, creating a proline residue which would disrupt α helical structure in the helices (18). These MyoD point mutations, H1pro and H2pro, also abolished the interactions with M-Twist (Fig. 4A, lanes 13 and 14) as well as with E proteins (18). Thus, in contrast to the truncated peptide bH1 (Fig. 3A, lane 13), a proper HLH structure in the whole MyoD molecule background is required for the interaction with M-Twist.

The unique residues in the MyoD basic domain are required for interaction with M-Twist. These data clearly illustrate a primary role of the MyoD basic domain in the interaction with M-Twist. The basic domains of bHLH proteins contain common amino acid residues as well as residues unique to each bHLH protein. We used the swap mutants of the MyoD basic domain to determine which residues are crucial for the interaction with M-Twist. We tested the three mutants, MyoD-E12B, MyoD-mycB, and MyoD-T4B, in which the MyoD basic domain was replaced with those of E12, c-myc, or drosophila achaete-scute T4, respectively (18). The basic domains of these bHLH proteins have some residues in common as well as some

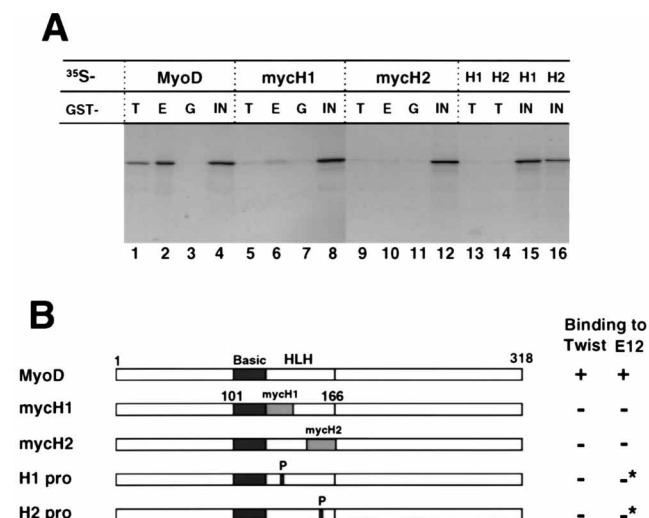
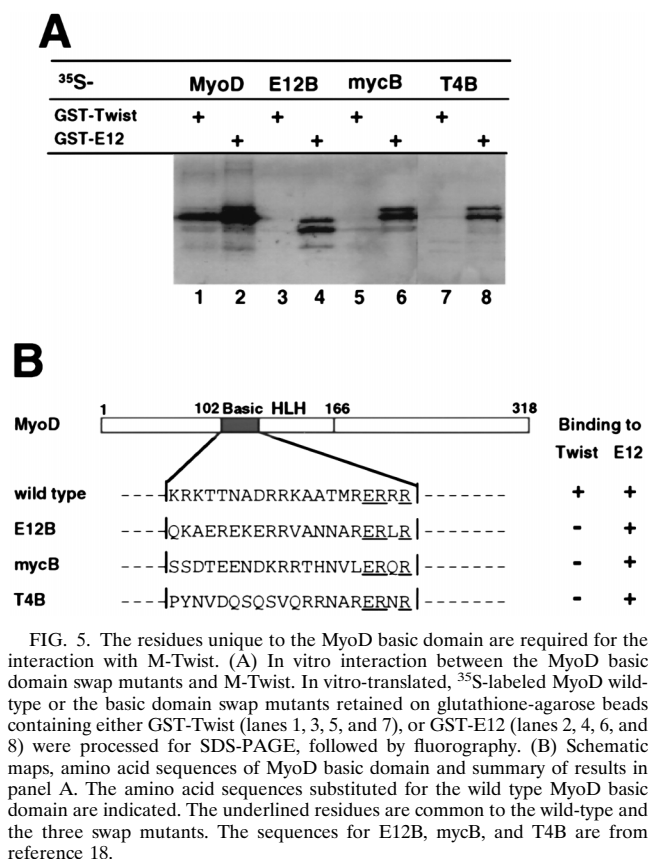


FIG. 4. The HLH structure of MyoD is required for the interaction with M-Twist. (A) In vitro interaction between the MyoD HLH domain mutants and M-Twist. In vitro-translated, ³⁵S-labeled MyoD HLH domain swap mutants (mycH1 and mycH2) or point mutants (H1pro and H2pro) retained on glutathione-agarose beads containing either GST-Twist (lanes 1, 5, 9, 13, and 14), GST-E12 (lanes 2, 6, and 10), or GST alone (lanes 3, 7, and 11), were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 4, 8, 12, 15, and 16). T, Twist; E, E12; G, GST alone; IN, input; H1, H1 pro; H2, H2 pro. (B) Schematic maps of wild-type MyoD and the HLH domain mutants and summary of results in panel A. *, data not shown, but reported by Davis et al. (18).



unique to each protein (Fig. 5B). We found that none of the three mutants interacted with M-Twist (Fig. 5A, lanes 3, 5, and 7), despite the retention of structural features required for interaction with E12 (Fig. 5A, lanes 4, 6, and 8 and 5B). These findings indicate that the residues shared by the different bHLH proteins cannot confer the interaction between MyoD and M-Twist and that this interaction requires unique residues within the basic domain of MyoD.

Myogenic residues within the MyoD basic domain are dispensable for interaction with M-Twist. To further define the amino acid residues of MyoD that are responsible for the interaction with M-Twist, different subregions spanning the MyoD basic and junctional regions were deleted (Fig. 6A). Amino acids 102 to 111 of MyoD are highly conserved among the myogenic bHLH proteins but are not present in the corresponding regions of E12, myc, or T4 bHLH proteins (Fig. 5B), suggesting that this region might contain residues that mediate specific interactions with M-Twist. Indeed, deletion of this subregion abolished the interaction with M-Twist (Fig. 6A, lane 5). Interestingly, the deletion of the middle segment, 112 to 116, which contains the myogenic code (alanine at 114 and threonine at 115) (19), did not affect the interaction (Fig. 6A, lane 8), suggesting that the myogenic code is not required for the interaction with M-Twist, although it is possible that M-Twist prevents recognition of these residues by other factors. The region from 117 to 121 is rich in arginine and well conserved among many otherwise unrelated bHLH proteins, including E12, myc, and T4 (Fig. 5B). Since neither MyoD-E12B, MyoD-mycB, nor MyoD-T4B can bind M-Twist (Fig. 5A, lanes 3, 5, and 7), we expected that amino acids 117 to 121 might be dispensable for interaction with M-Twist. Surprisingly, how-

ever, deletion of this region also abolishes interaction with M-Twist (Fig. 6A, lane 11). Deletion of the junction (ΔJ) showed only minimal reduction in the interaction whose functional significance remains to be clarified (Fig. 6A, lane 14). These findings defined two separate subregions, one conserved among the myogenic bHLH proteins and the other containing more common residues, which together may participate in the interaction with M-Twist.

The basic domain of M-Twist is required for interaction with MyoD. The unique interaction with M-Twist requiring the basic domain of MyoD prompted us to ask which domain of M-Twist is responsible for the interaction with MyoD. The deletion of part of the N terminus (ΔN) or C terminus (ΔC) of M-Twist did not cause drastic reduction in the interaction with MyoD (Fig. 7A, lanes 5 and 9). However, disruption of the HLH structure of M-Twist by deletion of the helix 2 and part of the loop (ΔBpu) abolished the M-Twist interaction with MyoD as well as its interaction with E12 (Fig. 7A, lanes 13 and 14). These data suggest that some or all of the bHLH domain of M-Twist is necessary for the interaction with MyoD. In accordance with these findings, the bHLH domain of M-Twist alone is capable of interacting with MyoD (Fig. 7B, lane 1). However, in the absence of the basic domain, the M-Twist HLH domain alone could not interact with MyoD, although it retained the structure required for interaction with E12 (Fig. 7B, lanes 3 and 4). Consistent with these observations, a deletion of the basic domain in a whole M-Twist molecule background abolishes the interaction with MyoD (Fig. 7B, lane 7), while its interaction with E12 is maintained (lane 8), indicating that the basic domain of M-Twist is required for interaction with MyoD. These results are summarized in Fig. 7C.

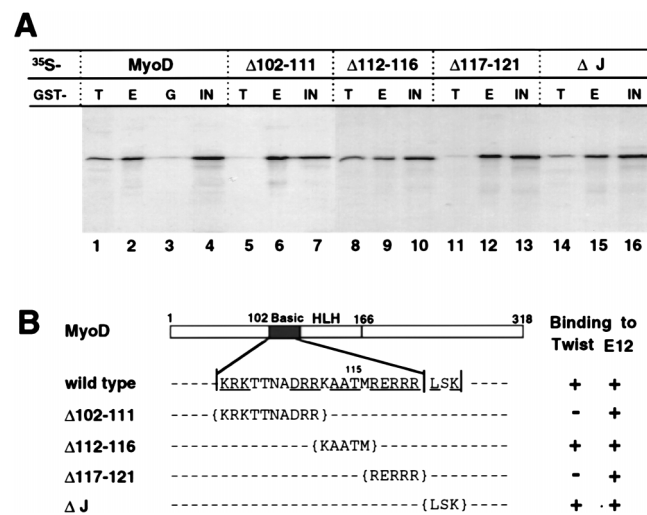


FIG. 6. Two MyoD basic domain subregions are required for the interaction with M-Twist. (A) Specific basic domain deletions in MyoD inhibit the interaction with M-Twist. In vitro-translated, ³⁵S-labeled MyoD basic and junctional region deletion mutants retained on glutathione-agarose beads containing either GST-Twist (lanes 1, 5, 8, 11, and 14), GST-E12 (lanes 2, 6, 9, 12, and 15), or GST alone (lane 3) were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 4, 7, 10, 13, and 16). T, Twist; E, E12; G, GST alone; IN, input. (B) Schematic maps, amino acid sequences of MyoD basic domain and summary of results in panel A. Vertical solid lines indicate the borders of different domains. The amino acid sequences of the MyoD wild type and the deleted residues (in brackets) of mutants are indicated. The residues that are invariant among other vertebrate species as well as other members of myogenic bHLH factors are underlined. Note that the region 112 to 116 containing the myogenic residues (alanine at 114 and threonine at 115) is not required for the interaction with M-Twist.

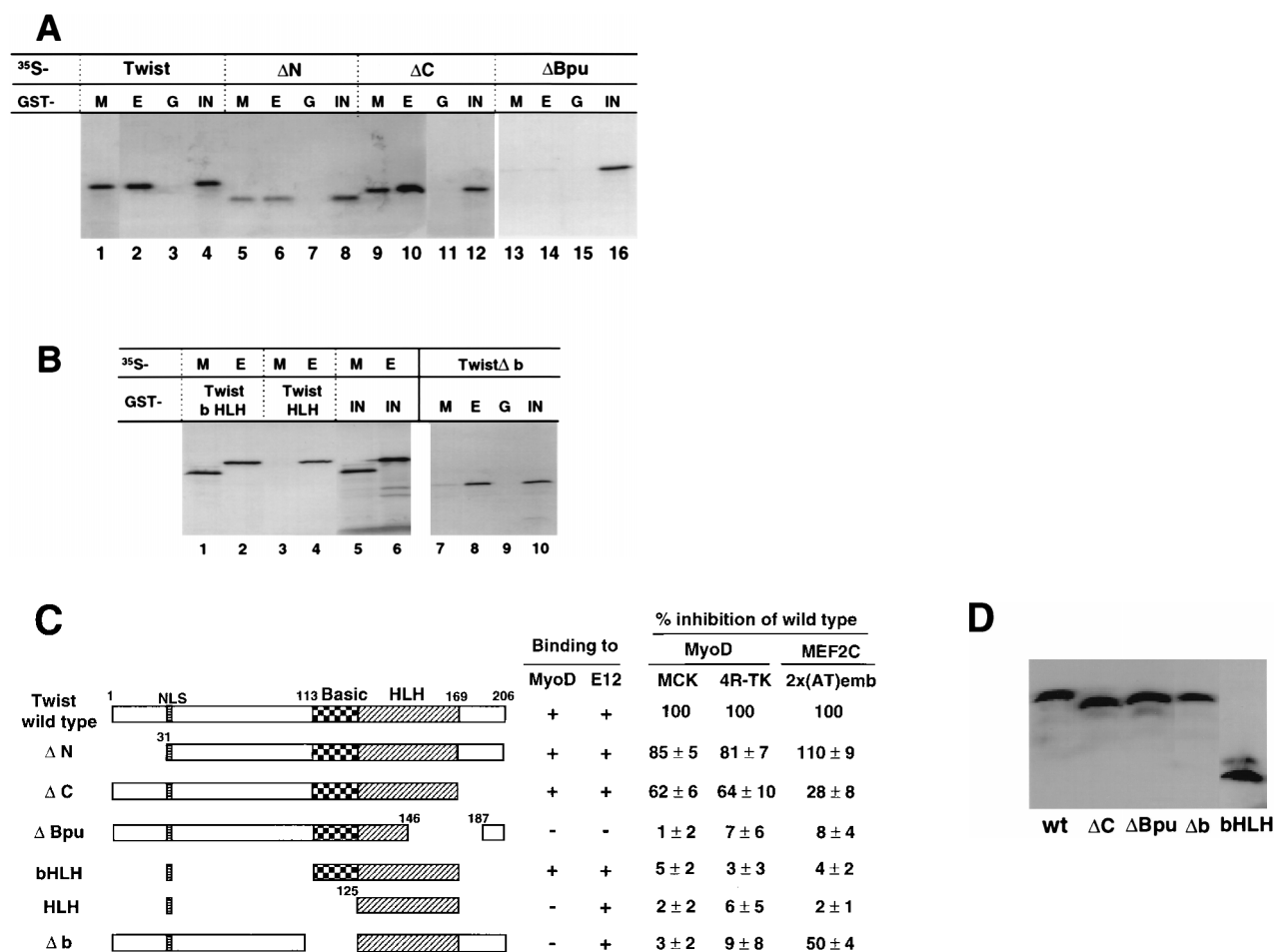


FIG. 7. M-Twist basic and HLH domains are required for the interaction with MyoD. (A) In vitro interaction between MyoD and the M-Twist deletion mutants. In vitro-translated, ³⁵S-labeled wild-type M-Twist (lanes 1 to 3), M-Twist-ΔN (lanes 5 to 7), ΔC (lanes 9 to 11), or ΔBpu (lanes 13 to 15) were incubated with glutathione-agarose beads containing either the GST fusions or GST alone as indicated at 4°C for 1 h. After extensive washing, bound proteins were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 4, 8, 12, and 16). M, MyoD; E, E12; G, GST alone; IN, input. (B) In vitro interaction between MyoD and M-Twist mutants. In vitro-translated, ³⁵S-labeled MyoD (lanes 1 and 3), E12 (lanes 2 and 4), or Twist-Δb retained on glutathione-agarose beads containing either GST-bHLH (M-Twist) (lanes 1 and 2), GST-HLH (M-Twist) (lanes 3 and 4), GST-MyoD (lane 7), GST-E12 (lane 8), or GST alone (lane 9) were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 5, 6, and 10). M, MyoD; E, E12; IN, input; G, GST alone. (C) Schematic maps of M-Twist and deletion mutants, summary of results in panels A and B, and the results of M-Twist functional studies. For the studies of MyoD inhibition by M-Twist, the 10T1/2 cells were cotransfected with either MCK-CAT (1 μg) or 4R-TK-CAT reporter and CMV-MyoD (0.5 μg) in the presence of the indicated M-Twist mutant expression vectors (0.2 μg). Fivefold excess of E protein expression vectors (CMV-E2-5 for MCK-CAT and Gal-E12 without an activation domain for 4R-TK-CAT) over the M-Twist vector was cotransfected in all the experiments to prevent E protein sequestration by M-Twist. The inhibition achieved by the wild-type M-Twist is described as 100% inhibition, and degrees of inhibition by different M-Twist mutants are described relative to that of the wild type. In parallel studies, inhibition by M-Twist of MEF2 transactivation of a MEF2-dependent promoter, pE102CAT[A/Tembx2], was analyzed. The 10T1/2 cells were cotransfected with the MEF2-dependent promoter-CAT (1 μg), CMV-MEF2C (2 μg), CMV-M-Twist (0.2 μg), and CMV-E2-5 (1 μg). We confirmed that MEF2C inhibition was observed only in the presence of the E protein vector, provided exogenously (59). Inhibition by the M-Twist constructs was similarly assessed. For the M-Twist bHLH and HLH mutants, the GST fusion proteins were used for the in vitro interaction study, and CMV-Twist-bHLH and -HLH with functional nuclear localization signals (NLS) were used for the functional studies. The basic domain of M-Twist is required for both interaction and inhibition of MyoD. The results are expressed as means ± standard errors from 4 to 6 independent experiments. (D) Various M-Twist mutants are expressed at comparable levels in 10T1/2 cells. 10T1/2 cells were transiently transfected with the expression vectors for myc epitope-tagged M-Twist, and the cell lysates (0.8 mg/lane) were subjected to SDS-15% PAGE, followed by Western blotting. The presence of the myc epitope did not affect functional data shown in panel C.

Functional implications of the interaction between M-Twist and MyoD. What is the functional role of the direct interaction observed between MyoD and M-Twist? We studied the ability of various M-Twist mutants to inhibit MyoD transactivation of MCK-CAT as well as 4R-TK-CAT, a simplified construct containing only multimerized preferred MyoD binding E boxes in the presence of an excess amount of E protein expression vectors (relative to the amount of the M-Twist vector) in order to alleviate E protein sequestration effects (59). Since the MyoD transactivation of MCK-CAT and possibly 4R-TK-CAT

(41) are likely to be a result of cooperative interaction between MyoD and MEF2C (34, 41), and M-Twist physically interacts with and inhibits MEF2 (27a, 59), M-Twist inhibition of MyoD could be attributed to the inhibition of MyoD and/or MEF2. To assess the relative contribution of MyoD versus MEF2 in the inhibition by M-Twist, we examined the effects of M-Twist on MEF2C transactivation of a reporter gene carrying two tandem MEF2 binding sites placed upstream of the embryonic myosin heavy chain basal promoter (pE102CAT[A/Tembx2]) (56). M-Twist strongly inhibited both the MyoD- and MEF2-

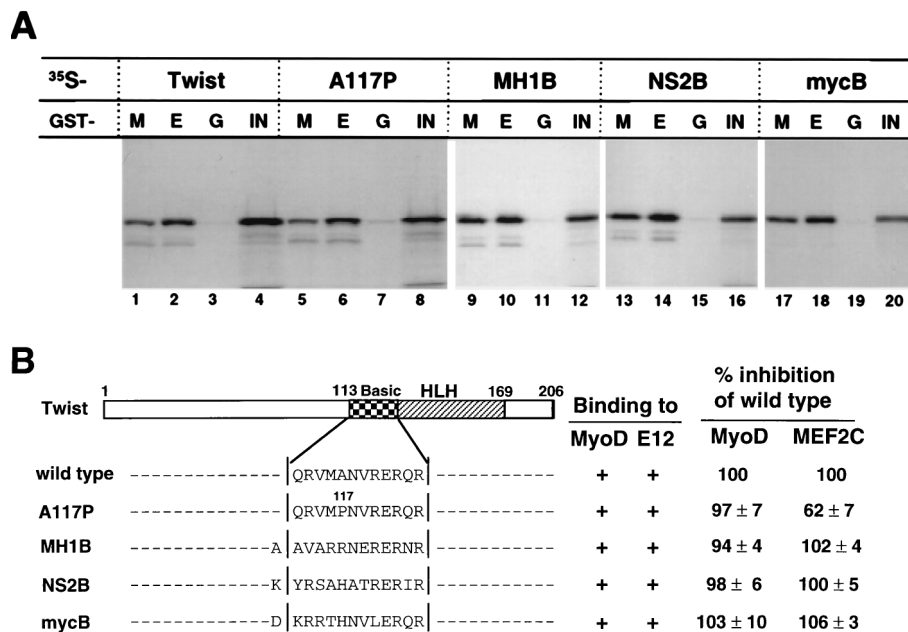


FIG. 8. M-Twist basic domains with substitutions from other bHLH proteins retain M-Twist functions. (A) In vitro interaction between MyoD and M-Twist basic domain mutants. In vitro-translated, ^{35}S -labeled wild-type M-Twist (lanes 1 to 3), a point mutant (lanes 5 to 7), or basic domain swap mutants (lanes 9 to 11, 13 to 15, and 17 to 19) were incubated with glutathione-agarose beads containing GST-MyoD (lanes 1, 5, 9, 13, and 17), GST-E12 (lanes 2, 6, 10, 14, and 18), or GST alone (lanes 3, 7, 11, 15, and 19) for 1 h at 4°C . After extensive washing, bound proteins were processed for SDS-PAGE, followed by fluorography. A117P, alanine at M-Twist position 117 replaced by proline. MH1B, NS2B, and mycB, M-Twist basic domain is replaced by those from mouse MASH1 (24), mouse NSCL2 (26), and the mouse c-myc (9), respectively. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 4, 8, 12, 16, and 20). M, MyoD; E, E12; G, GST alone; IN, input. (B) Schematic maps, amino acid sequences of M-Twist basic domain, summary of results in panel A, and M-Twist functional studies. The inhibition of MyoD and MEF2 transactivation by M-Twist is assessed as described in the legend for Fig. 7B. The results are shown as means \pm standard errors from four independent experiments.

dependent promoter constructs. ΔN had little effect on M-Twist inhibition of MyoD and MEF2 (Fig. 7C). ΔC , however, greatly reduced M-Twist inhibition of MEF2C transactivation, consistent with a previous report (59), although ΔC retains significant inhibition of MyoD. Disruption of the M-Twist HLH structure (ΔBpu) led to a loss of inhibition of MyoD in parallel with its inability to interact with MyoD. Neither the bHLH nor the HLH domain alone inhibited either MyoD or MEF2C transactivation (Fig. 7C), indicating that both the N and C termini of M-Twist contribute to its inhibition. Interestingly, the basic domain deletion mutant Δb failed to inhibit MyoD transactivation, consistent with loss of interaction with MyoD (Fig. 7C). To confirm that these M-Twist mutants were properly expressed in the cells, we performed immunoblotting, using lysates from transiently transfected cells, which showed comparable levels of protein expression (Fig. 7D). These findings illustrate a critical role of the M-Twist basic domain in both physical interaction with and inhibition of MyoD.

M-Twist basic domain swap mutants retain M-Twist functions. We next attempted to determine whether specific residues within the M-Twist basic domain were responsible for its interaction with and inhibition of MyoD. Surprisingly, substitution of a strong α helix-breaking proline for the alanine at amino acid position 117 (A117P) did not affect M-Twist interaction with MyoD (Fig. 8A, lane 5) or the inhibition of MyoD transactivation (Fig. 8B). This indicates that DNA binding is probably not required for MyoD inhibition by M-Twist, in agreement with a previous observation (59). To determine whether the residues involved in MyoD inhibition by M-Twist are unique to M-Twist, we tested the effect of several different swap mutations of the M-Twist basic domain. Surprisingly, we found that replacing the basic domain of M-Twist with those from MASH1 (MH1B), NSCL2 (NS2B), and c-myc (mycB)

had essentially no influence on binding to MyoD (Fig. 8A, lanes 9, 13, and 17) or suppression of MyoD or MEF2 transactivation (Fig. 8B). These findings suggested that common residues, rather than residues unique to the M-Twist basic domain, were likely to play the key role in the interaction with and inhibition of MyoD.

Common arginine residues in the M-Twist basic domain are required for interaction and inhibition of MyoD but not of MEF2C. To more precisely determine which M-Twist residues are involved in the interaction with and inhibition of MyoD, we tested several other M-Twist mutants. Deletion of the first several residues of the M-Twist basic domain ($\Delta 113-119$) resulted in a reduction of approximately 50% in the physical interaction with and inhibition of MyoD (Fig. 9A, lane 5 and 9B). Interestingly, replacement of the three common arginines at positions 120, 122, and 124 with alanines (R3-A3) abolished the ability of M-Twist to interact with and inhibit MyoD (Fig. 9A, lane 8 and 9B). In contrast to the loss of MyoD inhibition, the R3-A3 mutant retains both interaction with E12 and inhibitory activities on MEF2C transactivation (Fig. 9B). These findings reinforce the critical roles of the conserved basic residues (R120, 122, and 124) in the M-Twist basic domain. This observation simultaneously raises the question of the origin of the molecular specificity for target protein recognition. One candidate region is the loop segment (38, 50). We tested two loop region mutants, Twi-GPL and Twi-KLS. The Twi-GPL mutant, in which the initial five residues of the loop were replaced with glycine and proline, was almost as efficient as the wild type in interacting with MyoD as well as E12 and in inhibiting MyoD (Fig. 9A, lanes 11 and 12 and 9B). Twi-GPL, however, failed to inhibit MEF2C transactivation. The other loop mutation, Twi-KLS, in which the residues K, L, and S were replaced with glycine and proline, abolished all the tested

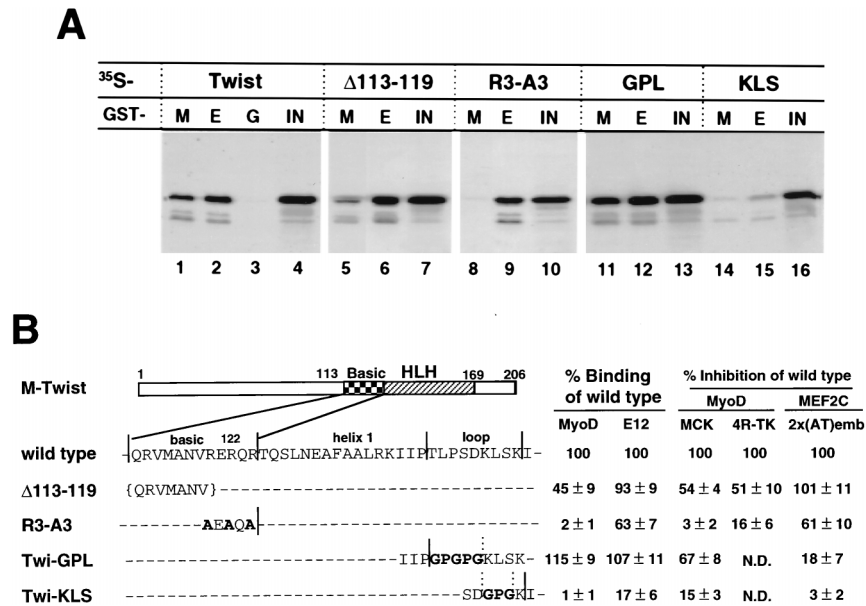


FIG. 9. The conserved arginine residues in the M-Twist basic domain are required for interaction with and inhibition of MyoD. (A) In vitro interaction between MyoD and M-Twist mutants. In vitro-translated, ^{35}S -labeled wild-type M-Twist (lanes 1 to 3), basic domain mutants (lanes 5, 6, 8, and 9), or loop region mutants (lanes 11, 12, 14, and 15) retained on glutathione-agarose beads containing either GST-MyoD (lanes 1, 5, 8, 11, and 14), GST-E12 (lanes 2, 6, 9, 12, and 15), or GST alone (lane 3) were processed for SDS-PAGE, followed by fluorography. Of the in vitro-translated products, 1/10 was loaded as an input (lanes 4, 7, 10, 13, and 16). M, MyoD; E, E12; G, GST alone; IN, input. (B) Schematic maps, amino acid sequences of M-Twist bHLH domain, summary of results in panel A, and M-Twist functional studies. Deleted amino acids are indicated by brackets. Vertical solid and dotted lines indicate the borders of different domains and the borders between the mutated regions and wild-type residues, respectively. The residues that were replaced are bold-faced. For analysis of the in vitro protein interaction data, the intensity of each band was measured by a densitometer. The intensity for each band was first determined relative to each construct's own input (percentage). The percentage of input for each construct was compared with that of the wild type and was expressed relative to that of the wild type, which was set at 100%. The results are shown as means \pm standard errors from four to six independent studies for both in vitro binding and transfection assays. The M-Twist residues required for MyoD inhibition differ from those for MEF2C inhibition, as indicated by R3-A3 and Twi-GPL mutants. N.D., not determined.

functions (Fig. 9A, lanes 14 and 15). We noted that some of the tested mutations, such as R3-A3 and Twi-GPL, displayed highly contrasting effects on MyoD and MEF2, suggesting that M-Twist inhibits these two transcription factors through distinct regions. Although the results obtained with the R3-A3 mutant indicate a critical role for this portion of the M-Twist basic domain in MyoD inhibition, the precise localization of the residues required for the specific recognition of MyoD remains to be determined.

DISCUSSION

M-Twist inhibition of myogenesis has previously been related to E protein sequestration and MEF2 inhibition (59). Although these mechanisms may be involved in the inhibition by M-Twist, there are two indications that these are not the sole mechanisms for the M-Twist inhibition. First, the overexpression of E proteins and MEF2 proteins cannot rescue inhibition of MyoD by M-Twist. Secondly, the MyoD-E47 tethered dimer is resistant to the E protein sequestration effect of Id, and its transactivation is independent of MEF2; yet, M-Twist efficiently inhibits this forced dimer (73). In the present study, we have demonstrated that M-Twist can directly interact with MyoD. This interaction represents a unique protein-protein interaction between two bHLH proteins of the class B group (cell type-specific bHLH proteins). Detailed mutational analysis identified the interaction between M-Twist and MyoD as being based on a novel strategy that requires the basic domains of both proteins. Basic domains are fully dispensable for the conventional HLH interaction between the class B group and E proteins (class A group) (18, 65). Since MyoD, E proteins, and MEF2 can form a ternary complex (34, 41), and

different domains of M-Twist can recognize these proteins (59; this study), the ternary complex might be a better in vivo target for M-Twist. Lack of recognition by the different M-Twist mutants of one or more of these protein components of the complex might result in inefficient interactions in vivo and the reduced inhibition of MyoD shown by these mutants. It must be emphasized that several M-Twist mutants showed preferential inhibition of MyoD relative to inhibition of MEF2 (and vice versa), suggesting that the MyoD inhibition is mechanistically separate from MEF2 inhibition. Importantly, the degrees of functional inhibition of MyoD by these mutants correlated with their activities in the physical interaction with MyoD. These data reinforce the concept of a role for direct M-Twist-MyoD interaction in MyoD inhibition by M-Twist.

The basic domains as essential components for interaction between M-Twist and MyoD. We have defined the two subregions in the MyoD basic domain that are required for the interaction with M-Twist (Fig. 6). The first region (102 to 111) contains residues conserved among the myogenic bHLH proteins, and the second region (117 to 121) contains arginine residues more common to bHLH proteins in general (Fig. 5B and 6B). Although it remains to be determined whether these residues contact M-Twist directly or simply provide an appropriate local conformation for other residues to contact M-Twist, the finding that the MyoD bH1 fragment can interact with M-Twist favors a direct interaction at the basic domain. The amino acids in the M-Twist basic domain required for the interaction with MyoD have been narrowed down to three basic residues (Fig. 9). Since these residues are invariable among most of the known bHLH proteins, it is likely that

additional residues are required for recognition of a specific target such as MyoD.

The oligomeric composition of the interacting proteins was not addressed in the present studies. While an M-Twist/E protein heterodimer is likely to be responsible for the inhibition of MEF2 by M-Twist (59), M-Twist homodimerization is readily detectable in vitro (Fig. 2, lane 5), and twist homodimerization has been reported in *Drosophila* (63). Thus, we cannot exclude the possibility that an M-Twist homodimer might interact with MyoD, which would result in a higher-order interaction. High-order interactions, such as tetramer formation among bHLH proteins, have been reported in studies of Id, MyoD, and myogenin in solution as well as with some class C bHLH proteins such as c-myc, USF, TFEB, and TFE3 (2, 3, 17, 20 to 23). It remains to be determined whether tetramer formation between MyoD and M-Twist is a part of MyoD regulation by M-Twist.

The basic domain of MyoD as a target of positive and negative regulators. Accumulating evidence supports the role of the MyoD basic domain in protein-protein interactions (7, 18, 19). Our findings provide evidence for the presence of a cellular factor, M-Twist, that negatively regulates MyoD through the basic domains. The unique aspect of the finding is that this regulator itself is a protein of the same bHLH class (class B) and that this interaction involves the basic domains of the two bHLH proteins. This type of interaction between basic domains of tissue-specific bHLH proteins appears to be unprecedented, although interactions between bHLH proteins with non-bHLH proteins have been demonstrated. For example, the MyoD and myogenin bHLH domains can interact with the leucine zipper domain of c-jun and serum response factor, respectively (8, 27). The bHLH domains of the myogenic bHLH proteins are also a target for the adenovirus E1A protein, which antagonizes myogenesis (12, 62). In these cases, it is possible that the basic domain of MyoD serves as an interface for the interaction. It has previously been proposed that the recognition of myogenic amino acids (alanine 114 and threonine 115) present in the MyoD basic domain by an as-yet-undefined mechanism leads to the exposure of an otherwise masked activation domain in the N terminus. There are several candidates for such a mechanism, including cofactors such as MEF2 (34, 41) and muscle LIM protein (35), which might recognize the myogenic residues in the MyoD basic domain. Although these myogenic residues were not required for the interaction with M-Twist (Fig. 6), M-Twist binding to the MyoD basic domain might interfere with the role of the myogenic residues. Although the N terminus of MyoD by itself does not interact with M-Twist in vitro (not shown), M-Twist might prevent the processes that lead to the exposure of the MyoD activation domain following the recognition of myogenic residues.

A basic DNA-binding domain is implicated in protein-protein interactions among various transcription factors, including factors containing helix-turn-helix motifs and zinc finger motifs (4, 47, 49, 64, 70, 75). The basic domains are in general the most conserved region among members of a single transcription factor family. Interaction at the basic domains might merely provide a contact site for specific interactions, while other regions serve as the effector domains. Alternatively, the interactions at the basic domains themselves may have intrinsic effector activity. However, since the M-Twist bHLH domain by itself can interact with MyoD but cannot inhibit MyoD transactivation (Fig. 7C), the interaction alone does not appear to be sufficient for inhibition. Although our data support the notion that the physical interaction is essential for the inhibition of MyoD by M-Twist, it is likely that additional functions

are provided by the N and C termini of M-Twist. M-Twist's direct physical interaction with MyoD might thus result in interference with processes such as the recruitment of other transcriptional factors (55, 67), association with coactivators (56, 72), and/or basal transcriptional factors.

The role of the Twist family of bHLH proteins in regulating cellular determination and differentiation. Myogenesis is extremely sensitive to extrinsic inductive cues (16). Such extrinsic signals are transduced to the nucleus in order to meet cellular and tissue requirements by regulating the proper onset of myogenic determination and differentiation and by ensuring the presence of a proper number of muscle cells at the appropriate positions in the body. It is well established that myogenesis is under both positive and negative regulation by proteins emanating from surrounding tissues, including Sonic hedgehog, various Wnt proteins, and BMP4 (16). The function of M-Twist that we observed in tissue culture should ultimately be reviewed in this context.

In the presomitic mesoderm, both M-Twist and a low level of the myogenic bHLH protein are detected (25, 48, 51, 58, 60, 71, 73). As somitic compartmentalization starts, M-Twist expression is first eliminated from the myotome, where high levels of myogenic bHLH proteins are expressed. Based on these expression patterns of M-Twist and myogenic bHLH proteins in the somite and on tissue culture data (28, 59; this study) it has been postulated that the primary role of M-Twist may be to prevent a premature onset of myogenic determination and/or ectopic myogenesis. Other members of the Twist family, such as dermo-1 and paraxis, which have similar but significantly different temporal and spatial expression patterns, might have similar functions (11, 13, 39, 52). The results of studies with M-Twist knockout mice as well as those of studies with tissue cultures are also consistent with functional redundancy among the family members (15, 39). We hypothesize that some of these members of the Twist family use the common basic domain protein interaction strategy. Since these proteins are also expressed in other sites, such as sclerotome and neural crest cells, they might participate in the regulation of cellular determination and in the differentiation of cell types other than muscle.

Our data support the view that M-Twist may regulate proper onset of cell fate determination and/or further differentiation and thus ensure the generation of the required number of muscle cell precursors at the appropriate times in the correct positions. To execute this critical task, M-Twist may employ several mechanisms. In this study, we have proposed a role of the unique basic domain interaction strategy that would involve the regulation of tissue-specific bHLH proteins by M-Twist with more specificity.

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ADDENDUM IN PROOF

At the time of submission, a related study was reported (M. Hebrok, A. Fuchtbauer, and E.-M. Fuchtbauer, *Exp. Cell Res.* **232**:295–303, 1997).

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